Rusinowski, S, Krzyżak, J, Clifton-Brown, J, Jensen, E, Mos, M, Webster, R, Sitko, K and Pogrzeba, M

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Environmental Pollution 252 (2019) 1377–1387

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

New *Miscanthus* hybrids cultivated at a Polish metal-contaminated site demonstrate high stomatal regulation and reduced shoot Pb and Cd concentrations☆

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ARTICLE INFO

Article history:
Received 1 April 2019
Received in revised form
12 June 2019
Accepted 15 June 2019
Available online 20 June 2019

Keywords:
Gas exchange
Stomata
Water use efficiency
Heavy metal

ABSTRACT

The increased bioeconomy targets for the biomass share of renewable energy production across Europe should be met using land unsuitable for food production. Miscanthus breeding programs targeted the production of plants with a diverse range of traits allowing a wider utilization of land resources for biofuel production without competing with arable crops. These traits include increasing tolerances to drought, chilling, and to metal(loid)s excess. Two novel Miscanthus hybrids, *GNT41* and *GNT34,* were compared against *Miscanthus* x *giganteus* (*Mxg*) on metal-contaminated arable land in Poland. This study aimed at evaluating their yield, biomass quality and quantifying seasonal differences in photosynthetic and transpiration parameters. A secondary objective was to identify key physiological mechanisms underlying differences in metal accumulation between the investigated plants. The new hybrids produced a similar yield to *Mxg* (13–15 t ha^{-1} yr^{-1}), had shorter shoots, higher Leaf Area Index and stem number. Based on gas exchange measurements, *GNT34* exhibited isohydric (water-conserving) behavior. The stomatal response to light of the new hybrids was at least twice as fast as that of *Mxg*, a trait that is often associated with increased seasonal water use efficiency. This contributed to the almost 40% reduction in shoot Pb and Cd concentrations for the new hybrids as compared to *Mxg*. This suggested that promoting stomatal regulation in conjunction with improved water conservation may be a target for improving plants for wider use on metals contaminated land.

1. Introduction

Renewable energy and bioeconomy targets are set to increase market demand for lignocellulosic biomass in Europe (Scarlat and Banja, 2013). These targets could be met using marginal lands unsuitable for food production. An estimated 13 million hectares of European agricultural topsoils exceed potentially safe limits for metal(loid) contamination (2009/28/EC; EC 1881/2006; Tóth et al., 2016), recognizing however that total soil contents are not indicating metal(loid) phytoavailability. Replacing food crops with non-food perennial lignocellulosic biomass crops on such land could deliver multiple environmental, economic and social benefits. Direct benefits include a reduction in foodstuffs contaminated with metal(loid)s due to cessation of food crop cultivation on such land, the creation of alternative enterprises for regional rural development, and achieving renewable energy targets prescribed by the European Commission in RES Directive (Scarlat and Banja, 2013; Kidd et al., 2015). Such practice also brings phytostabilization benefits, where contaminated run-off into surrounding water bodies is reduced through transpiration and better water infiltration into the root zone, and erosion of contaminated particulates is mitigated through dense roots and rhizomes.

☆ This paper has been recommended for acceptance by Prof. Wen-Xiong Wang.
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https://doi.org/10.1016/j.envpol.2019.06.062

Both woody and herbaceous perennial biomass crops are candidates for metal(loid)-contaminated lands. The most appropriate choice of crop depends on local environmental conditions and markets associated with local biomass processing chains. One leading candidate for metal(loid)-contaminated land in Poland, with its continental climate of warm summers and cold winters, is the Asian grass *Miscanthus*. *Miscanthus* is a close relative of tropical sugarcane, using the same C4 photosynthesis that increases efficiencies in radiation, water and nitrogen use. However, unlike sugarcane, *Miscanthus* tolerates much cooler European and temperate climates (Głowacka et al., 2014).

Miscanthus spp. are outbreeding, occurring over a wide climatic range throughout eastern Asia, and thus providing extensive genetic diversity for improvement through breeding. Commercial cropping currently relies on clones of the naturally occurring hybrid *Miscanthus* × *giganteus* (*Mxg*) (Greef and Deuter, 1993; Hodkinson and Renvoize, 2001) which, despite arising naturally, has many attributes that are hard to improve upon, including yields exceeding those of common switchgrass and maize cultivars in US studies (Dohleman and Long, 2009; Heaton et al., 2008). However, use of such a limited genetic base presents a disease risk, and establishment is costly because *Mxg* is a sterile triploid, requiring vegetative propagation (Lewandowski, 1998). Due to various environments and end uses, i.e. combustion vs. conversion to liquid fuel, the introduction of much more diverse germplasm is likely required. In addition, cold and drought tolerance in *Mxg* are inferior to some other genotypes (Clifton-Brown et al., 2015; Dohleman and Long, 2009; Fonteyne et al., 2018; Głowacka et al., 2014; Jones and Walsh, 2001; Sage et al., 2015).

Since 2011, breeders at the Institute of Biological, Environmental and Rural Sciences (IBERS) have produced hybrids of *M. sinensis* and *M. sacchariflorus* in an effort to provide alternatives to the clonal triploid *Mxg* (Greef and Deuter, 1993) and overcome limitations associated with clone-based crops by producing seed-based hybrids. High water use efficiency is important for drought-prone marginal land types and in such areas, shorter stature hybrids may be more resilient and produce higher average yields than the taller *Mxg* types (Clifton-Brown et al., 2002). Another key trait may be the 'stay-green' phenotype (Thomas and Howarth, 2000), observed by Clifton-Brown et al. (2002) in an interspecific hybrid, which also exhibited delayed leaf senescence and the highest whole season production under drought conditions. Targets related to drought resilience might be resolved using varieties exhibiting isohydric behavior, rather than anisohydric, by reducing the risk of xylem cavitation during prolonged drought (for review see; Martínez-Vilalta and Garcia-Forner, 2017).

In 2014, novel seeded hybrids from the IBERS breeding programme showed early promise at several European locations, and in 2015 these trials were extended to three sites in Poland, including one near Katowice where temperature extremes are common, and total soil metal concentrations exceed limits for arable land. Until recently, Katowice was the centre of the Polish smelting industry, where metals, pumped through smoke stacks into the atmosphere, were deposited onto soil surfaces for over a century. Levels of Pb, Cd and Zn in the 0–30 cm topsoil (ploughed layer) are about 700 mg kg^{-1}, 25 mg kg^{-1} and 3000 mg kg^{-1}, respectively (three-fold, eight-fold and six-fold above safe limits for arable lands according to Polish Regulation Dz.U, 2016 poz. 1395, respectively). Sustainable and economically productive alternative land uses are therefore required.

Literature describing the physiological impacts of metal(loid) excess on *Miscanthus* spp. is limited (Guo et al., 2016; Kocoń and Jurga, 2017; Nsanganwimana et al., 2016; Pogrzeba et al., 2017), with still fewer investigations regarding seed-based hybrids (Bang et al., 2015; Krzyżak et al., 2017). This study aimed at evaluating yield and biomass quality of two novel interspecific *Miscanthus* seed-based hybrids against the standard *Mxg*, grown on metal contaminated land, and quantifying seasonal differences in photosynthetic and transpiration parameters. A secondary objective was to identify key physiological mechanisms underlying differences in metal accumulation between the investigated plants.

2. Materials and methods

2.1. Trial design

A field trial was established on arable land contaminated with Pb, Cd and Zn from metal smelting at the Bytom site (50°20′ 43.0″N 18°57′ 19.6″ E) near Katowice, Poland. For the last 20 years, cereals have been cultivated there (Pogrzeba et al., 2017). The trial design was a complete randomized block including three replicates, each containing plots (25 m^2, 2 plants per m^2) of two novel interspecific *Miscanthus* seed-based hybrids (*M. sinensis* x *M. sacchariflorus GNT41* and *GNT34*) and the commercial standard *Miscanthus* x *giganteus* (*Mxg*) (Krzyżak et al., 2017). *Miscanthus* seed-based hybrid plugs were provided by Terravesta Assured Energy Crop Ltd. (UK) and *Mxg* rhizomes were delivered by Energene Sp. z o.o. *Miscanthus* seed-based hybrids were bred by Aberystwyth University in the UK during the GIANT LINK Program (LK0863). No fertilization or plant protection products were applied on the field. Manual weeding was done only at the beginning of the first growing season. Plant survival was assessed before the start of the second growing season (2016) and expressed as a percentage of plant survival per plot. Weather data were collected daily from a meteorological station located about 10 km from the trial. Soil moisture deficits were calculated as the accumulative deficit between daily rainfall and the modelled evaporation rates. Daily rainfall and evapotranspiration rates (calculated by a spreadsheet version of the Penman-Monteith equation (Hess and Stephens, 1993) were used to estimate the daily accumulative soil moisture deficit. When the soil moisture deficits were lower than plant available water in the soil profile (150 mm) then the soil was water limited.

2.2. Analysis of soil physico-chemical properties

Five soil cores per plot were sampled to a depth of 20 cm before the trial was established (time zero samples). Soil samples from each plot were combined, air dried and sieved through 2 mm and 0.25 mm, for soil analysis. Soil (<2 mm) pH was analyzed in H_2O and 1 M KCl (ratio 1:2.5 m/v) with a combination glass/calomel electrode (OSH 10-0, METRON, Poland) and pH-meter (CPC-551, Elmetron, Poland) at 20 °C, while soil electrical conductivity (EC) was obtained using an ESP 2 ZM electrode (EUROSENSOR, Poland) on the same device according to Polish norm PN-ISO 11265:1997.

Soil *aqua regia* extracted Cd, Pb and Zn (<0.25 mm) were analyzed using flame atomic absorption spectrometry (SpektrAA 300, Varian INC., USA) after hot plate digestion in *aqua regia,* according to the ISO 11466:1995 (Pogrzeba et al., 2017).

The phytoavailable Cd, Pb and Zn concentrations in the topsoil (<0.25 mm) was assessed using soil extraction with 0.01 M $CaCl_2$ following methods described in Pogrzeba et al. (2017). Filtrates were analyzed using flame atomic absorption spectrometer (SpektrAA 300, Varian INC., USA).

2.3. Analysis of gas exchange and plant pigment contents

Monthly measurements from June to September of instantaneous photosynthesis, stomatal conductance and transpiration rate

during second growing season (2016) were performed between 8a.m. and 1p.m. with an infra-red gas analyzer (LCpro SD, ADC Bioscientific, UK) on the youngest fully expanded leaves of two shoots, on three marked plants per plot. The LCpro SD was equipped with a narrow chamber (580 mm^2), set at 22 °C, 1500 μmol m^{-2} s^{-1} for photosynthetically active radiation (PAR), and with ambient CO_2 levels of ca. 400 ppm. Intrinsic water use efficiency (iWUE) was calculated as a quotient of light saturated photosynthesis rate to stomatal conductance (Webster et al., 2016). In addition, following McAusland (2016), stomatal conductance and photosynthetic response curves for dark adapted leaves exposed to saturating light intensities were performed in the field during the night on 18th of June 2018 (forth growing season, drought stress). The ambient temperatures and CO_2 levels on this night were 19 °C and 420 ppm. The humidity was low, resulting in a vapour pressure deficit of 4 KPa. Each response curve was created by logging the photosynthetic parameters every 10 s with an Infra-Red Gas Analyzer (IRGA) as the incident PAR was stepped from darkness after 4 min to full saturating light intensity of 1500 μmol m^{-2} s^{-1} for 15 min and then back to darkness for 5 min (McAusland, 2016).

Chlorophyll content (μg cm^{-2}), Anthocyanins Index (relative units) and Flavonols Index (relative units) were measured using a plant pigment content meter (Dualex Scientific+, Force-A, France) on the same leaves as for gas exchange measurements in the 2016 growing season. Additionally, the Dualex device provided a Nitrogen Balance Index (NBI).

2.4. Determination of biomass yield and plant traits

At the end of September (2016), Leaf Area Index (LAI) measurements were carried out using a Ceptometer (LP-80, Decagon device, USA) for each plot. The LAI were estimated for the whole plot using the mean of four independent measurements, using an external PAR sensor as a reference. At the end of the second growing season (October 2016), traits were measured on three adjacent plants in a centre row of each plot. Shoot height was determined as the distance from the ground to the ligule of the tallest stem. Stem count was determined as the number of shoots per plant with a height equivalent to, or exceeding, 60% of the tallest shoot. This effectively eliminates small insignificant shoots that are not contributing to the canopy and the biomass yield (Kalinina et al., 2017). Senescence was estimated using the ratio of 'canopy die off height' (height from the ground to point where leaves are green) divided by the shoot height. In autumn (October 2016), shoots were harvested with a hedge trimmer 10 cm above the soil surface on quadrats of 4 m^2 (8 plants). The shoot fresh weight (f.w.) yield from the whole quadrat was determined in the field on a hanging balance. Subsamples of ~200 g f.w. were separated into leaves and stems, weighed, then surface washed with deionized water before being dried to a constant weight for 72 h at 70 °C. The dried subsamples were milled for chemical analysis to <1 mm. Specific Leaf Area (Reich et al., 1998) was calculated on the plot basis based on the earlier obtained data as follow:

$$Leaf : Shoot \left[\frac{g}{g}\right] = leaf\ d.w.\ [g] \div (leaf\ d.w.\ [g] + stem\ d.w.\ [g]) \quad (1)$$

$$Leaf\ biomass\ per\ m^2 \left[\frac{kg}{m^2}\right] = Leaf : Shoot \left[\frac{g}{g}\right] \times Biomass\ yield \left[\frac{kg}{m^2}\right] \quad (2)$$

$$SLA \left[\frac{m^2}{kg}\right] = LAI \left[\frac{m^2}{m^2}\right] \div Leaf\ biomass\ per\ m^2 \left[\frac{kg}{m^2}\right] \quad (3)$$

2.5. Determination of metal concentration in shoots

Dried shoot samples were milled and hot plate digested (1 g d.w.) in the solution of nitric and perchloric acid (4:1 v/v) (Schierup and Larsen, 1981). Concentrations of Pb, Cd and Zn in the plant shoots were analyzed in digested samples using flame atomic absorption spectroscopy (SpektrAA 300, Varian INC., USA).

2.6. Statistical analysis

All analyses were performed using Statistica 13.1 (Dell, US). Statistical differences were estimated by ANOVA tests at the 5% level ($P \leq 0.05$) of significance for all parameters evaluated. Where ANOVA indicated a significant difference, a post-hoc Fisher's least significant difference (LSD) test was carried out. Correlation analyses were performed using Pearson's correlation for selected parameters. All presented means were expressed with ±SE.

3. Results

3.1. Site characteristics

The climatic conditions during the experiment (T: 15 °C; precip: 481 mm) were similar to the average values on the previous 69 years (T: 17 °C; precip: 446 mm) for the same period, during the growing season (Fig. S1), except for July and August when plants were exposed for short periods to temperatures exceeding 30 °C. No significant soil water deficit (SWD) was identified during the 2016 growing season (Fig. S2). Table 1 shows the soil physico-chemical properties for the field trial. Spatial variabilities of pH_{H20}, pH_{KCl}, electrical conductivity (EC), and total and bioavailable Cd and Zn were detected across the plots, but these were not statistically significant (Table 1). In all soil samples $CaCl_2$-extractable Pb concentrations were below detection limits (<0.07 mg kg^{-1}).

3.2. Gas exchange parameters

Mxg had the highest rates of light saturated photosynthesis (A_{sat}) over the whole growing season and were, on average, 30%

Table 1
Soil physico-chemical properties in Bytom, Poland (0–20 cm depth).

	Mxg	*GNT41*	*GNT34*
physico-chemical parameters			
pH_{H20}	6.5 ± 0.03a	6.52 ± 0.05a	6.54 ± 0.07a
pH_{KCl}	5.99 ± 0.02a	5.99 ± 0.05a	6.02 ± 0.04a
EC (μS cm^{-2})	83.1 ± 0.3a	80.9 ± 3.9a	75.4 ± 2.4a
Total soil metals			
Pb (mg kg^{-1})	689.5 ± 30.9a	621.4 ± 4.4a	719.6 ± 25.1a
Cd (mg kg^{-1})	26.5 ± 2.36a	23.2 ± 0.65a	26.5 ± 0.80a
Zn (mg kg^{-1})	3028 ± 228a	2590 ± 58a	3312 ± 276a
0.01 M $CaCl_2$-extractable metals			
Pb (mg kg^{-1})	Bdl	Bdl	Bdl
Cd (mg kg^{-1})	1.48 ± 0.08a	1.23 ± 0.04a	1.57 ± 0.18a
Zn (mg kg^{-1})	102.3 ± 5.46a	86.6 ± 3.53a	111.2 ± 17.1a

Values are means ± SE (n = 3).
Lower case letters (a, b, c) denote significant differences among soils samples taken from different plots at $P \leq 0.05$ according to Fisher LSD test.
Mxg – *Miscanthus* x *giganteus*, EC - Electrical conductivity.
Bdl – below detection limit (<0.07).

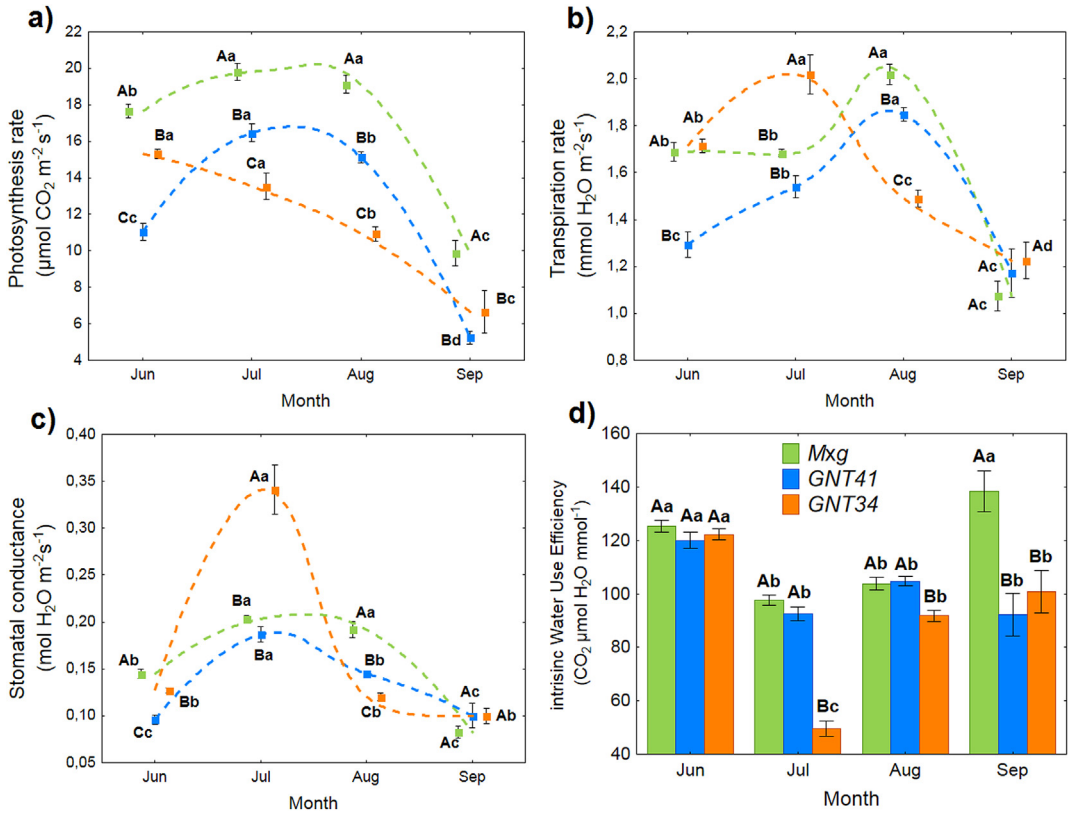

Fig. 1. Leaf gas exchange parameters showing a) maximum assimilation at saturating light (A_{sat}), b) leaf transpiration (E), c) stomatal conductance (g_s), and d) instantaneous water use efficiency (A_{sat}/g_s), measured monthly from June to September for interspecific *Miscanthus* seed-based hybrids (*GNT41* and *GNT34*) and *Miscanthus* x *giganteus* (*Mxg*) grown on heavy metal contaminated arable land in Bytom, Poland. Values are means ± 1 SE (n = 54). Upper case letters (A, B, C) indicate least significant differences (Fisher LSD) at $P \leq 0.05$ between hybrids within a measurement month. Lower case letters (a, b, c) refer to significant differences between different months in the same hybrid.

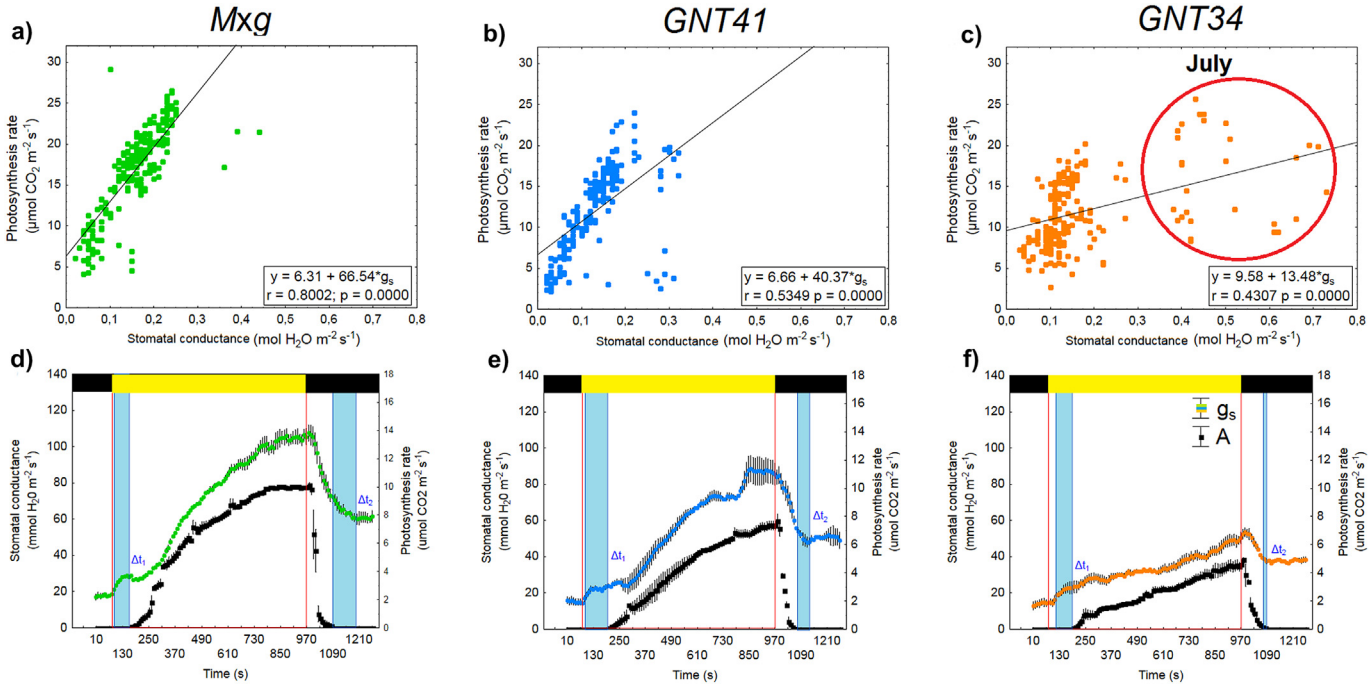

Fig. 2. Regression of light saturated (1500 µmol m^{-2} s^{-1}) stable rates of photosynthesis with stomatal conductance for (a) *Miscanthus* x *giganteus* (*Mxg*) (b), *GNT41*, and (c) *GNT34* from monthly infrared gas analysis measurements made between 9 and 10 a.m. during 2016, and dynamic responses of stomatal conductance (coloured symbols) and photosynthesis (black symbols) rates with transitions from darkness to saturated light levels for 15 min (970 s) and back to darkness (d) *Mxg* (e), *GNT41*, and (f) *GNT34*. All measurements in (d) to (f) were made during the night (midnight to 3 a.m.) on 18th of June 2018. Values are means ± 1 SE (n = 3). Δt_1 – is the time lag between light 'switch on' (stomatal opening) and the start of carbon assimilation, Δt_2 - is the lag between a cessation of carbon assimilation and stomatal closure.

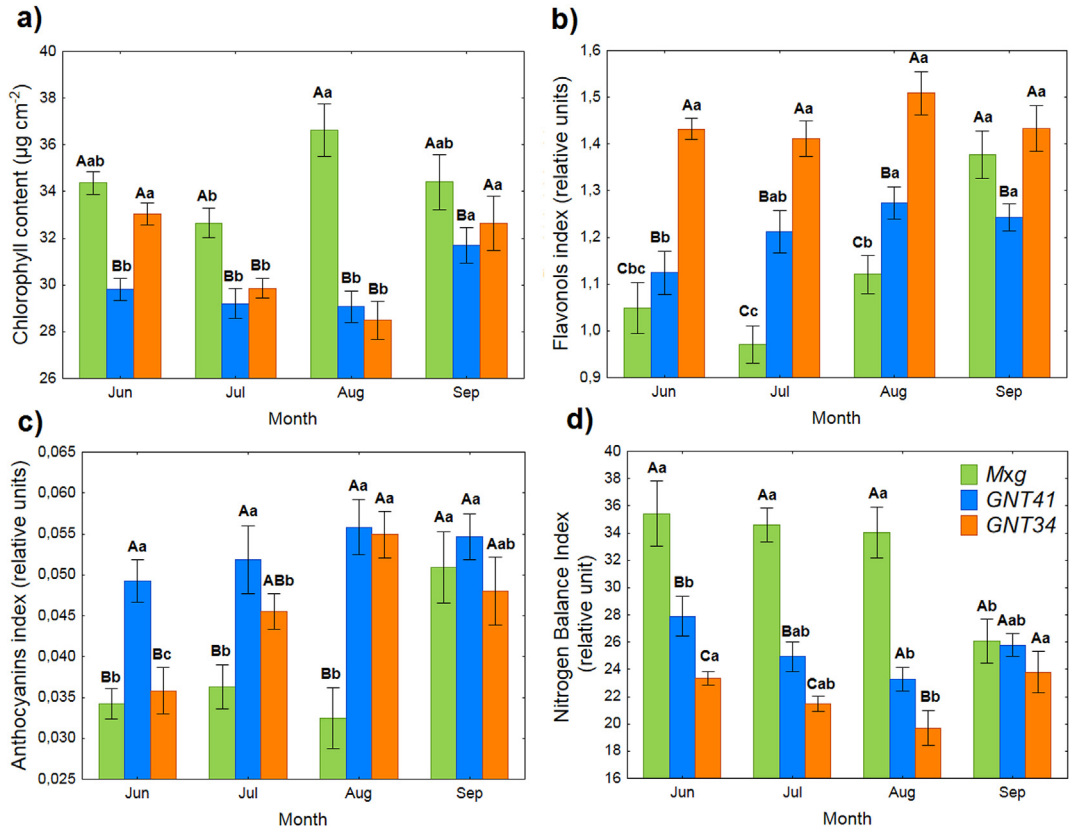

Fig. 3. Chlorophyll content (a), Flavonols index (b), Anthocyanins index (c) and Nitrogen Balance Index (d) measured in June, July, August and September for interspecific *Miscanthus* seed-based hybrids *GNT41* and *GNT34* and *Miscanthus* x *giganteus* (*Mxg*) cultivated on heavy metal contaminated arable land. Values are means ± SE (n = 36). Upper case letters (A, B, C) indicate least significant differences (Fisher LSD) at $P \leq 0.05$ between genotypes within a measurement month. Lower case letters (a, b, c) refer to significant differences between different months in the same genotype.

above those of *GNT34* and *GNT41* (Fig. 1a). Rates of photosynthesis peaked in June for *GNT34*, and in July for *GNT41* and *Mxg*. Interestingly, the rate for *GNT34* decreased steadily from June to September. In contrast, a decline in A_{sat} for *Mxg* and *GNT41* occurred from August onwards.

Average transpiration rate (Fig. 1b) measured in June, July, August and September was at a similar level (1.5–1.7 mmol H_2O m^{-2} s^{-1}) for all investigated genotypes. As for photosynthesis, *GNT34* showed a different seasonal pattern to *GNT41* and *Mxg*. There were no significant differences in transpiration between *Mxg* and *GNT34* in June, but in July *GNT34* showed the highest transpiration rates, which then fell below *Mxg* and *GNT41* in August. No significant differences between transpiration rates of the three cultivars were detected in September.

Average stomatal conductance (Fig. 1c) was the highest for *GNT34* in July, when values for *Mxg* and *GNT41* were 12% and 23%, respectively, below *GNT34*. Conductance followed the seasonal patterns of transpiration for *GNT34*, with peak rates in July. *Mxg* retained higher rates in August than *GNT41* and *GNT34*, but by September all cultivars had similarly low rates.

Intrinsic water use efficiency (iWUE, Fig. 1d) was the highest for *Mxg* and *GNT41* in July and August, while in June obtained iWUE values were similar for all investigated plants. In September *Mxg* showed the highest iWUE compared to the hybrids.

Relationships between the rate of photosynthesis and stomatal conductance (Fig. 2) presented as all measurement which were taken during the 2016 growing season between June and September for *Mxg* (Fig. 2a), *GNT41* (Fig. 2b) and *GNT34* (Fig. 2c) showed significant correlation between photosynthesis rate and stomatal conductance irrespectively of genotype, although the correlation strength and slope of the regression differed between genotypes. These differences were particularly associated with measurements taken in July.

Table 2
Shoot metal concentrations, plant traits and green biomass yield at the end of the second growing season (September–October 2016).

	Mxg	*GNT41*	*GNT34*
Shoot DW yield and plant traits			
Survival rate* (%)	96.7 ± 1.3a	79.3 ± 3.5b	97.3 ± 0.7a
Shoot yield (kg DW m^{-2})	1.38 ± 0.45a	1.46 ± 0.24a	1.23 ± 0.11a
Moisture content (%)	62.37 ± 1.14b	64.75 ± 1.01a	64.83 ± 1.13a
Shoot height (cm)	300 ± 6a	266 ± 7b	200 ± 11c
Stem count per plant	17 ± 2c	33 ± 3b	39 ± 1a
Die off height (%)	15.67 ± 0.02	ND	ND
Leaf:Stem**	0.34 ± 0.02b	0.69 ± 0.09a	0.58 ± 0.03a
Leaf:Shoot**	0.26 ± 0.01b	0.40 ± 0.03a	0.37 ± 0.01a
LAI (m^2 m^{-2})	2.30 ± 0.21b	3.01 ± 0.24a	2.85 ± 0.30a
SLA (m^2 kg^{-1})	8.15 ± 2.64a	5.53 ± 1.41a	6.34 ± 0.76a
Shoot metal concentrations			
Pb (mg kg^{-1})	38.08 ± 2.67a	30.53 ± 1.99b	22.25 ± 2.27c
Cd (mg kg^{-1})	0.84 ± 0.09a	0.52 ± 0.03b	0.47 ± 0.05b
Zn (mg kg^{-1})	245.8 ± 9.2a	194.6 ± 15.2b	213.1 ± 18.6 ab

Values are means ± SE (n = 9), except biomass, LAI, SLA, survival rate where values are means ± SE (n = 3).
Lower case letters (a, b, c) denote significant differences among plant samples and measurements taken from different genotypes at $P \leq 0.05$ according to Fisher LSD test.
Mxg – *Miscanthus* x *giganteus*. ND – not detected, *after first winter from the establishment, **biomass ratio.

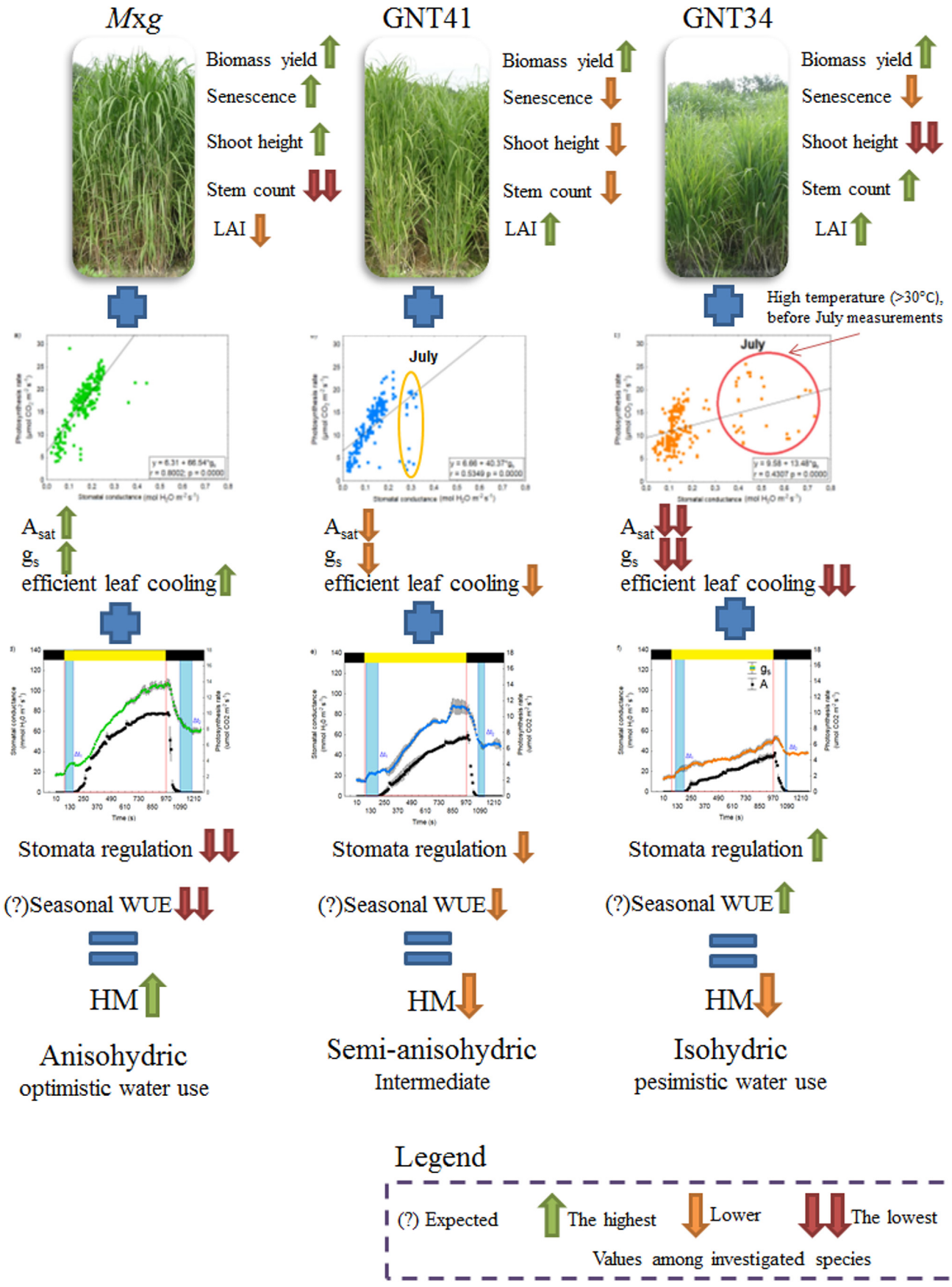

Fig. 4. Proposed mechanism showing a set of traits and interactions between them which might be associated with the lower/higher heavy metal accumulation in above-ground biomass during the whole growing season. All investigated hybrids which produce similar biomass yields differ among phenotypic and photosynthetic traits. The parameters which

The response of dark-adapted stomata to applied light (Fig. 2d–f) showed that stomatal conductance was 23% and 56% lower in *GNT41* and *GNT34,* respectively, than for *Mxg* (about 110 mmol H_20 m^{-2} s^{-1}). Interestingly, the time taken to plateau after light was turned off was twice as long (4 min) for *Mxg* than for *GNT41* (2 min) and almost instantaneous for *GNT34*.

3.3. Plant pigment content and nitrogen balance index

Relative chlorophyll content (Fig. 3a) was the highest for *Mxg* at all measuring points while *GNT41* and *GNT34* showed similarly low values in July, August and September. However in June, chlorophyll content was significantly higher (about 14%) in *GNT34* than in *GNT41*. Overall, *GNT34* showed decreasing values between June and July with an increase between August and September. Chlorophyll content of *GNT41* remained unchanged in June, July and August but significantly increased (10%) in September. Chlorophyll content in *Mxg* peaked in August.

Flavonols index (Fig. 3b) increased through the growing season for *Mxg* and *GNT41*, but remained relatively static for *GNT34*. The latter had the highest flavonol index, about 1.45 r.u., followed by *GNT41* and *Mxg*, the only anomaly being in September, where similar values were found for *Mxg* and *GNT34* and significantly lower values (about 11%) for *GNT41*.

Anthocyanin values (Fig. 3c) for *Mxg* were consistently low in June, July, August, with a 56% increase in September, whilst remaining unchanged across the measuring period for *GNT41*. Values for *GNT34* increased from June to August.

The nitrogen balance index (NBI, Fig. 3d) showed the same pattern between genotypes from June to July, with highest values in *Mxg*, and 25% and 37% lower values in *GNT41* and *GNT34*, respectively. In September, there were no significant differences between species, as *Mxg* had fallen by 24%.

3.4. Shoot yield and plant traits

Mxg and *GNT34* had almost 100% winter survival, whilst a lower survival rate was found for *GNT41* (79%) (Table 2). An inverse relationship between shoot height and stem count resulted in similar yields for all hybrids (Table 2). The moisture content of *Mxg* was significantly lower than that of *GNT34* and *GNT41,* which reflected senescence values (Table 2). These were measured as the proportion of the crop height that had fully senescence (aka 'die off height'), divided by the total plant height (Table 2). Seeded hybrids had nearly double the leaf to stem d.w. ratios of *Mxg,* which was partially reflected in LAI values (Table 2). Specific Leaf Area (SLA) did not significantly differ among investigated plants, which may be influenced by changes in yields and LAI across plots of the same species, however higher values for *Mxg* were evident.

3.5. Shoot metal concentrations

Shoot ionome revealed higher Pb, Cd and Zn concentrations for *Mxg* plants as compared to *GNT41* and *GNT34* (by 46%, 68% and 20%, respectively). No significant differences were found for shoot Cd and Zn concentration between *GNT41* and *GNT34*, while shoot Pb concentrations for *GNT34* were about 28% lower than for *GNT41* (Table 2).

4. Discussion

A major requirement for any perennial crop such as *Miscanthus* is winter survival following the first growing season (Clifton-Brown and Lewandowski, 2000). Survival rates above 70% are commercially acceptable (Anzoua et al., 2015). Previous work on the same field showed two out of four hybrids planted (in addition to *Mxg*) did not survive the first winter (Krzyżak et al., 2017). Both *Mxg* and *GNT34* had almost 100% winter survival rates and over 80% of *GNT41* plants survived.

Throughout the growing season *GNT34* exhibited different photosynthetic responses compared to *GNT41* and *Mxg*, particularly with respect to the timing of maximum rates of saturated photosynthesis, transpiration, and stomatal conductance. This could be due to higher developmental plasticity in *Mxg* and *GNT41* as compared to *GNT34*. Developmental plasticity may be limited to early stages of the life cycle, and may also vary in timing among these genotypes (Sultan, 2000), additionally faster growing plants are more plastic in their growth responses than slower growing types (Bret-Harte et al., 2001). Our observations support these findings, as *GNT34* was characterized by shorter stems and lower shoot biomass as compared to the *GNT41* and *Mxg*.

Based on data obtained during a drought period (3 weeks without rainfall, Fig. S1) in the 2018 growing season, stomatal conductance in both *Mxg* and *GNT41* was unchanged (Fig. 1d,e,f), however stomatal conductance in *GNT34* was reduced by about 60% when compared to the same month in the 2016 growing season (Fig. 1a,b,c). This provides evidence that *GNT34* expresses isohydric stomata behavior. This behavior characterizes plants that express a conservative water use strategy; such plants control stomatal conductance to reduce water loss through leaf transpiration. This often leads to reduced photosynthetic rates. In contrast, plants with anisohydric behavior, use soil water resources until lower water potentials are attained. This can result in faster xylem cavitation under drought conditions. However, anisohydric behavior leads to higher evapo-transpiration, reducing leaf temperatures. While this protects leaves from heat stress, it is costs water. It also increases the risk of damage from xylem cavitation (Chaves et al., 2016). Joo et al. (2017) reported that *Mxg* expresses more anisohydric traits than other perennial C4 grasses (i.e. *Panicum virgatum*). To underline observed differences, regression between photosynthesis rate and stomatal conductance for *GNT34* showed decoupling of those parameters in July when high maximum air temperatures (over 30 °C) were recorded. According to Urban et al. (2017), there is a stable relationship between photosynthesis rate under light saturated conditions (A_{sat}) and stomatal conductance at low temperatures. However, that relationship can breakdown at high temperatures. In addition, due to un-sustained period of high temperatures, decoupling of A_{sat} and g_s were not observed in the following months, suggesting that *GNT34* has the capability to recover from moderate heat-stress. Earlier and ongoing physiological studies have indicated that stomata remain open in *Mxg* even when plant water potentials are below −30 bar, which is twice the standard 'wilt point' of 15 bars (Clifton-Brown et al., 2002). These studies show that *Mxg* plants, during periods of water deficit, continue to maintain high transpiration rates at the top of the canopy while lower leaves rapidly senesce.

The decrease of chlorophyll content during growing season is a commonly used indicator of the senescence (Mos et al., 2013; Purdy

were responsible for lower accumulation of heavy metals were associated with low height, high stem count, late senescence and higher leaf area index (LAI). Moreover those plants, during the growing season expressed a lower light saturated photosynthesis rate (A_{sat}) and stomatal conductance (g_s) with less efficient leaf cooling mechanisms, associated with decoupling of g_s from A_{sat} at high temperature when compared to *Mxg*. Moreover an additional factor was the faster response of stomata to changing environment, which may control water loss and transpiration more efficiently, which may result in higher whole season water use efficiency (seasonal WUE). These parameters indicate that species with less optimistic water use strategies (isohydric behavior) might accumulate less heavy metals, during an "optimal" growing season.

et al., 2015). Dualex data collected on the last fully expanded leaf showed that *Mxg* has the highest chlorophyll content out of the hybrids in the study.

Both seed-based hybrids expressed higher flavonol and anthocyanin indices (except September) when compared to *Mxg*. Besseau et al. (2007) reported that *Arabidopsis thaliana* (L.) Heynh. with silenced of hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT), which leads to the biosynthesis of two major lignin building units, showed increased flavonoid content simultaneously, inhibiting plant height probably due to inhibition of auxin transport in plants. Moreover silenced plants showed significantly lower lignin content. Relationship between flavonoid content and auxin transport was also previously reported (Brown et al., 2001; Gayomba et al., 2016). The shorter phenotype could be driven by lower lignin content in the plant structure, as reflected by the higher flavonoid content in *Miscanthus* seed-based hybrids. Those associations were also supported by Hodgson et al. (2010) who analyzed lignin content in many *Miscanthus* genotypes including *Mxg* and interspecific hybrids between *M. sacchariflorus* and *M. sinensis*. *Mxg* showed higher lignin content when compared to both *M. sinensis* and interspecific hybrids. This observation was also valid for plants investigated in this experiment (A. Kiesel, personal communication). Interestingly, lignin content varied between experimental locations in different climate zones, hence further investigation is needed to generalize lignin levels for novel hybrids (Hodgson et al., 2010).

The decreasing values between August and September of 'die off height' and NBI for *Mxg* suggests that *GNT41* and *GNT34* are more related to "stay-green" genotypes (Thomas and Howarth, 2000) compared to *Mxg*. In addition, the flavonol and anthocyanin indices were not significantly affected by the appearance of newly developed leaves during the growing season at the different measuring points, which was found for chlorophyll contents. This suggests that the NBI, which is a proximate indicator of C:N allocation changes (Cerović et al., 2012), could be a more valuable tool for estimating the rate of senescence than the relative chlorophyll content (Mos et al., 2013), irrespective of the changes in the leaf position during the growing season. Additionally, the changes in gas exchanges in September suggest that the delayed senescence, as expressed by chlorophyll content, in *GNT41* and *GNT34* is not reflected by the maintenance of photosynthesis. The possible explanation of delayed senescence could be associated with the endogenous cytokinins and ABA concentration in xylem sap (Dong et al., 2008). During senescence, endogenous signals up-regulate certain genes that show high homology to enzymes known to degrade protein, RNA, lipids, and chlorophyll (Buchanan-Wollaston, 1997). However, factors involved in delayed senescence should be further investigated.

Clifton-Brown et al. (2002) found similar tendencies for *Mxg* and a "stay-green" genotype, which had higher stem density and shorter shoots. The expression of lower ABA concentration was linked to drought resilience in that genotype. This response may also be true for other plant species. Lafitte et al. (2006) reported that semi-dwarf rice genotypes were more resilient to drought stress. This was caused by a reduction in gibberellin content in the plant and the interaction between gibberellin and ABA.

The highest Intrinsic Water Use Efficiency (iWUE) was evident in *Mxg* irrespective of the measuring time point, however this was not always a significant response. This result does not detract from the drought resilience expressed by *GNT41* and *GNT34*, because plants were not exposed to water deficits during the 2016 growing season (Fig. S2, SMD). Based on photosynthesis and transpiration measurements, Clifton-Brown et al. (2002) reported that *Mxg* is probably better suited to short periods of drought, whilst "stay-green" genotypes may produce better yields during sustained periods of drought.

Due to the nature of *Miscanthus*' perenniality, it is usually considered mature after the 3rd growing season (Anderson et al., 2011). There was no statistical difference between the shoot d.w. yield of ca. 14 tons ha^{-1} attained for the three genotypes in 2016 (second growing season) as compared to 15–16 tons ha^{-1} (data not shown) in 2017. These results are similar to those of more established plantations in similar geographical ranges as the Bytom site, of 9–14 Mg ha^{-1} yr^{-1} (Lewandowski et al., 2016).

Despite *GNT34* consistently having the lowest light saturated photosynthesis rate per leaf area, the three genotypes produced a similar amount of biomass. This could be driven by higher Leaf:-Stem biomass ratio as well as the higher Leaf Area Index (LAI) for *GNT41* and *GNT34*. The highest *Mxg* light saturated photosynthesis rates may also be associated with the highest SLA. Jiao et al. (2016) reported correlation between higher photosynthesis rates and SLA in different *Miscanthus* species and accessions. This shows that having thinner leaves is not necessarily at the expense of a reduced assimilation rate. Moreover, Dohleman et al. (2009) showed that *Mxg* had a higher SLA than Switchgrass, and simultaneously, a higher photosynthesis rate. However, those plants which express a lower SLA are somewhat more drought resilient (Costa-Saura et al., 2016; Zhang et al., 2015), providing further evidence for the drought resilient characteristics expressed by the two new hybrids.

Shoot metal concentrations were highest for the *Mxg* plants compared to the seed-based hybrids. This was most evident for the non-essential elements (Pb and Cd) that do not maintain physiological functions in plants (Nagajyoti et al., 2010). Slight differences occurred in shoot Zn concentrations. These shoot metal concentrations were not above the upper critical threshold levels for higher plants (Pb: 300 mg kg^{-1}, Cd: 10 mg kg^{-1}, Kabata-Pedias, 2010), as evidenced by few detectable impacts on the physiological status of the genotypes. Despite this, the decrease of the non-essential metal concentrations in the shoot biomass of the hybrids, in comparison to *Mxg*, is highly desirable. These shoot Pb, Cd, and Zn concentrations may not be problematic in the lignocellulosic biomass biochemical-conversion technology, such as anaerobic digestion (Mudhoo and Kumar, 2013), or bioethanol production (Xie et al., 2014). However, lower non-essential metal concentrations in harvested biomass will undoubtedly improve the management of residues after any conversion processes (Pogrzeba et al., 2018).

Both *Miscanthus* seed-based hybrids exhibited a more rapid response of stomatal closure in response to changing light conditions. After the light source was turned off the stomata of *Mxg* remained open for ca. 2 min. The stomatal response of *GNT41* was somewhat more rapid than *Mxg*, whilst the stomatal closure of *GNT34* was almost instantaneous when the light source was turned off. Lawson and Blatt (2014) explained that manipulating the stomatal response to changing environmental conditions could provide a means to both improve the water use efficiency of plants and at the same time increase the integrated photosynthetic carbon gain. This inconspicuous feature regarding stomatal response could significantly affect plant water use efficiency diurnally, mostly due to changes in the irradiation (e.g. cloud flow). When considered across a whole growing season this could make a substantial difference to carbon uptake and water loss. Moreover, measurements of iWUE in a steady state environment do not fully reflect the water use efficiency experienced in a field environment; steady-state situations are rarely, if ever, observed in nature (Lawson and Blatt, 2014). The importance of the relationship between leaf transpiration rate and stomatal regulation in relation to metal accumulation is well studied (Liu et al., 2010; Liu et al., 2016; Salt et al., 1995; Tassi et al., 2008).

Shoot metal concentrations, notably for non-essential metals

such as Pb and Cd, is sometimes assumed to reflect a mass flow transport from the soil matrix into the stems and leaves through transpiration (Salt et al., 1995). Accumulation in plant shoots appears to be controlled by several processes including root uptake, radial transport, xylem loading, and translocation from the roots to the shoots. Transpiration is a driver for bulk water flow in this process. From 66% to 82% of the relationship between Cd concentration in field crops and in the soil solution could be explained by the amount of transpired water (Ingwersen and Streck, 2005). Tassi et al. (2008) reported that application of exogenous cytokines, one of their functions being the regulation of stomatal opening, resulted in increased transpiration rate and consequently the flux of water soluble contaminants to the upper parts of plants. Moreover, Salt et al. (1995) reported that exposure of *Brassica juncea* to ABA caused large increase in the stomatal diffusive resistance of leaves, and this was associated with a dramatic reduction in the accumulation of Cd in the leaves. To stress the importance of the transpiration in those processes it was found that pretreatment of hyperaccumulator *A. halleri* with ABA dramatically decreased the proportion of ^{109}Cd and ^{65}Zn distributed to shoot (Zhao et al., 2006). It has been suggested that more than one physiological process controls translocation from soil solution to plant tissues. For this reason, additional factors such as differences in the gene-expression associated with the ion transporters to which metals (Pb, Cd) have affinity, e.g. Nramps, HMAs, CDF family, ZIP family, could not be excluded (Hall and Williams, 2003). It would be a logical step to suggest that the reduction of shoot metal concentration would be associated with plants that exhibit isohydric rather than anisohydric behavior. Additionally, the importance of the stomatal response time could be considered crucial, which is supported by the relative concentrations of Pb and Cd in the biomass across the genotypes studied (*Mxg* > *GNT41* > *GNT34*). From this we may postulate that plants with more efficient WUE, rapid stomatal responses and that express more isohydric traits, may be targeted for selection. Plants with these traits should yield biomass that is less contaminated during cultivation on Pb and Cd contaminated lands, while simultaneously improving photosynthesis rate, water use efficiency, and aid plant productivity (Lawson and Vialet-Chabrand, 2019). Some of the possible mechanisms and interactions responsible for the differences in shoot metal concentration across the genotypes studied is provided as a graphic in Fig. 4. A high useable yield is the primary criteria for any biomass crop. The new hybrids studied here show that there are new *Miscanthus* varieties that, despite growing on contaminated (or other idle) land, can maintain a competitive yield, with reduced shoot metal concentrations and with higher resource use efficiency. We recommend further investigations to take into account a wider range of Miscanthus hybrids in the presence of metal(loid) and water stress conditions.

5. Conclusions

This field trial of three *Miscanthus* hybrids grown on metal (Pb, Cd, Zn) contaminated land in southern Poland demonstrated annual shoot d.w. yields of 13–16 t dm ha^{-1} following the second and third growing seasons.

GNT41 and *GNT34* plants differed significantly in plant traits, retained higher green leaf areas and had lower shoot Pb and Cd concentrations than standard *Mxg* plants.

GNT34 exhibited isohydric behavior as compared to *Mxg* and *GNT41*. This was reflected in a significant reduction in stomatal conductance during a drought period (2016 growing season vs 2018 growing season). Moreover, lower metal accumulation in *GNT41* and *GNT34* was found. More dynamic transpiration, particularly in *GNT34*, linked to stomata that responded faster to light, and were more sensitive to internal water status. This resulted in increased seasonal water use efficiency.

Hybrids with improved drought resilience possess lower SLA and isohydric behavior resulting from more dynamic stomatal responses. These responses may become future targets in breeding for crop improvement because they reduce transpiration, making them more favorable for harsh metal contaminated environments. Further trials on metal(loid)-contaminated lands are recommended with the newest *Miscanthus* hybrids with proven stomatal regulation under drought stress to prove findings described in this paper.

The potential for expansion of high yielding *Miscanthus* hybrids with combined drought resilience and fast stomatal response on metal contaminated lands could supply biomass feedstock to expand the bioeconomy from marginal lands exceeding the safe limits for food crops.

Authors' contributions

S.R. (60%) - designed and maintained most of the experiment, statistical analyses, wrote first draft of the manuscript, **J.K., M.P.** (8%) - experiment design, statistical analysis, wrote first draft of the manuscript. **K.S.** (4%) – maintained the experiment. **J.C-B., M.M.** (5%) – contribution to experiment design, manuscript writing. **E.J., R.W.** (5%) – contribution to manuscript writing. All authors gave final approval for publication.

Conflicts of interest

The authors declare that they have no conflict of interest.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

Acknowledgement

Research financed by The Polish National Center for Research and Development (grant agreement No. FACCE SURPLUS/MISCOMAR/01/16) under the flag of Era-Net Cofund FACCE SURPLUS, in the frame of the Joint Programming Initiative on Agriculture, Food Security and Climate Change (FACCE-JPI). EJ also acknowledges funding from the European Regional Development Fund through the Welsh Government and Hefcw Sêr Cymru National.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.06.062.

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